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SYNTHETIC PEPTIDE VACCINES FOR THE CONTROL OF
ARENAVIRUS INFECTIONS(U) SCRIPPS CLINIC AND RESEARCH
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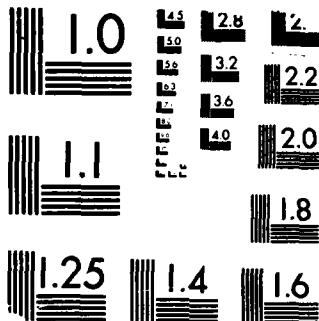
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SYNTHETIC PEPTIDE VACCINES FOR THE CONTROL OF
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INFECTIONS

ANNUAL REPORT

August 1, 1986 - July 31, 1987

M. J. BUCHMEIER and PETER SOUTHERN

July 31, 1987

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

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segments of LCMV and mapped the important immunogenic regions of the viral glycoproteins. The LCMV genomic RNAs have been cloned and primary sequences of the RNAs and their gene products are being completed. Synthetic peptides corresponding to immunogenic regions of the viral structural proteins are being synthesized and will be evaluated for the ability to induce immune responses in experimental animals. Experimental approaches to immunization based upon synthetic peptides and polypeptides, vaccinia virus vectors containing LCMV genes, and anti-idiotypic antibodies will be explored. Experimental approaches to immunotherapy for acute arenavirus infections will also be investigated using cloned cytotoxic T-lymphocytes and neutralizing monoclonal antibodies in attempts to modify the course of acute disease. Finally, monoclonal antibodies and cDNA probes against defined type specific and common determinants and sequences will be made in order to facilitate precise diagnosis of arenaviral diseases.

Abstract

Arenaviruses are endemic on both the African and South American continents and represent significant public health hazards. Prophylactic immunization, precise diagnostic methods, and effective treatment protocols are not currently available. We are using genetic cloning methods to develop an effective vaccine against arenaviruses. Developmental studies have been carried out and techniques established with the prototype arenavirus, lymphocytic choriomeningitis virus (LCMV), and these methods can now be applied for rapid development and evaluation of vaccines against the pathogenic arenaviruses Lassa, Junin, and Machupo. Using techniques of peptide and immunochemistry we have identified and mapped the gene products of the L and S-RNA segments of LCMV and mapped the important immunogenic regions of the viral glycoproteins. The LCMV genomic RNAs have been cloned and primary sequences of the RNAs and their gene products are being completed. Synthetic peptides corresponding to immunogenic regions of the viral structural proteins are being synthesized and will be evaluated for the ability to induce immune responses in experimental animals. Experimental approaches to immunization based upon synthetic peptides and polypeptides, vaccinia virus vectors containing LCMV genes, and anti-idiotypic antibodies will be explored. Experimental approaches to immunotherapy for acute arenavirus infections will also be investigated using cloned cytotoxic T-lymphocytes and neutralizing monoclonal antibodies in attempts to modify the course of acute disease. Finally, monoclonal antibodies and cDNA probes against defined type specific and common determinants and sequences will be made in order to facilitate precise diagnosis of arenaviral diseases.

Foreword

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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A. Introduction and Overview

During the past contract year we have focused on two major issues related to the work described in contract proposal C6234. These are:

1) Precise identification of antigenic determinants conserved among the arenaviruses. Previous studies have shown that pathogenic and non-pathogenic arenaviruses share antigens in both glyco- and nucleocapsid proteins. We have explored the molecular basis for this homology using synthetic peptides and monoclonal antibodies and have identified a short segment of nine amino acids of the GP-2 which contains one such conserved antigenic site.

2) Molecular cloning of LCMV and comparison with other arenaviruses. LCMV forms the prototypical arenavirus. As described in the final report for contract C3013 we recently completed sequencing of the S RNA of LCMV-Armstrong. In the past year we have extended those efforts to draw comparisons between LCMV and other arenaviruses, and to begin to correlate structural changes in S and L encoded products with differences in pathogenic potential among viruses.

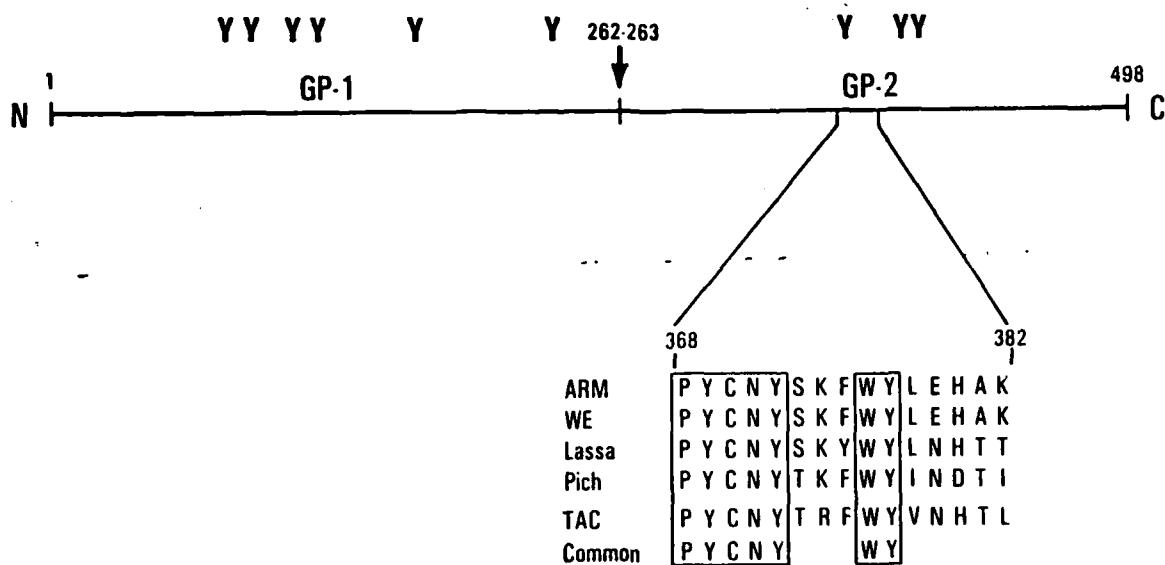
These studies have been described in detail in six publications listed in Appendix 1.

B. Precise identification of antigenic determinants conserved among arenaviruses

Toward the goal of precisely identifying antigenic determinants conserved among arenaviruses, we have localized and characterized a major antigenic site on the GP-2 glycoprotein of LCMV (Weber and Buchmeier, 1987, in press). Lymphocytic choriomeningitis virus S RNA encodes an intracellular precursor glycoprotein GP-C (76k) which is posttranslationally cleaved to yield the structural glycoproteins GP-1 (44k) and GP-2 (35k). Previous work from this laboratory has demonstrated that the GP-1 bears a single conformationally sensitive epitope against which neutralizing monoclonal and polyclonal antibodies are directed. GP-2, in contrast, contains an immunodominant epitope which is resistant to denaturation and a subset of antibodies to this epitope react broadly with heterologous arenaviruses of both the Old World (LCM, Lassa, Mopeia) and New World (Tacaribe complex) subgroups. We have mapped, to the level of amino acid sequence, the conserved epitope on GP-2 and find immunoreactivity of poly- and monoclonal antibodies against this protein confined to a synthetic peptide representing 13 amino acids spanning residues 370-382 of GP-C. Specificity was demonstrated by direct ELISA binding assays to the synthetic peptide, and by inhibition of binding of monoclonal and polyclonal antibodies to both native and denatured GP-2 by peptide. Comparisons of GP-C sequences of LCMV, Pichinde and Lassa viruses (Fig. 1) revealed a high degree of

homology among these viruses in residues 370-377 toward the amino terminus of the peptide wherein 6 of 8 amino acids are identical among these viruses. In contrast the 5 carboxyl terminal amino acids of this peptide are not conserved. The epitope appears to be of biological significance since polyclonal antisera against LCMV, Junin, and Lassa viruses all react with peptide 370-382.

Fig. 1 Identification of a Conserved Antigenic Site on LCMV GP-2



We have recently completed fine mapping of this conserved GP-2 epitope. Briefly, the binding site of promiscuous GP-2 specific monoclonals (33.6 and 83.6), that is, those which react broadly with all arenaviruses, as well as MAb 9-7.9 which reacts with LCMV and Mopeia virus only, have been mapped to a stretch of 9 amino acids spanning residues 370-378 of GP-C (Fig. 2):

Fig. 2

| | 370 | | | | | | | | | | 378 |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|---|-----|-----|
| LCM | Cys | Asn | Tyr | Ser | Lys | Phe | Trp | Tyr | | | |
| Lassa | . | . | . | . | . | Tyr | . | . | . | . | |
| Pic | . | . | : | Thr | . | . | . | . | . | . | |
| Tac | . | . | . | Thr | Arg | . | . | . | . | Tyr | |

As is evident from the sequences shown, this region is highly conserved across both Old and New World arenaviruses. From assays of polyclonal human and experimental animal antisera we have found that the same epitope accounts for up to 70% of the reactivity of a polyclonal antiserum against GP-2. LCM convalescent hyperimmune guinea pig and Junin late convalescent human sera were assayed for binding activity against GP-2 by Western blotting. We found that preincubation with the synthetic peptide in solution reduced by more than 70% the binding of these polyclonal sera against GP-2.

The molecular basis of limited specificity of MAb 9-7.9 was also elucidated. This MAb, which recognizes LCMV and Mopeia but not Lassa virus, was assayed for reactivity against the following peptide sequences corresponding to LCMV and Lassa respectively:

Fig. 3

| | | |
|-------|---------------|-----|
| | 370 | 382 |
| LCM | CNYSKFWYLEHAK | |
| Lassa | CNYSKYWYLEHAK | |

While the broadly reactive MAb 33.6 reacted with both sequences equally well, 9-7.9 reacted only with the LCM sequence (EIA titer of >1:10,000 vs <1:160). Thus substitution of Phe 375 with Tyr rendered the antibody nonreactive. These findings demonstrate the molecular identity of a native epitope on the LCMV GP-2 glycoprotein and the precise nature of the sequence difference between LCMV and Lassa conferring species specificity to the 9-7.9 monoclonal. Furthermore the existence of two epitopes within a 9 amino acid segment is proven.

In more preliminary experiments attempting to map the major neutralizing domain of the arenaviruses we have analyzed the primary nucleotide sequences of two strains of LCMV which differ in reactivity with a strongly neutralizing antibody. Strain ARM 4 is neutralized by neutralizing monoclonal antibody (MAb) 2-11.10 made in mice, and numerous other MAb from rats. Strain ARM 5 in contrast is not neutralized by these antibodies, nor does it bind antibody. To determine the molecular basis for this difference, and to begin to define neutralization determinants, we have sequenced, using the dideoxy chain termination method, the relevant glycoprotein GP-1 of each virus. Three amino acid differences were observed in the deduced amino acid sequences of the GP-1 glycoproteins of these viruses. These are summarized below:

Fig. 4

| | | | | |
|-------|---|--|-------------|-----|
| | 170 | | 185 | 252 |
| ARM 4 | -Y N L A F S D R Q S A Q S Q C R- | | -I L F S Q- | |
| ARM 5 | ... T ... R L . . | | | |

Of these changes the A → T change at position 173 is particularly interesting since it results in the insertion of a glycosylation site NLT. We are currently constructing synthetic peptides covering this region to screen them 1) for reactivity with neutralizing antibody, and 2) to attempt to raise neutralizing antibodies by peptide immunization.

C. Molecular cloning of LCMV and comparison with other arenaviruses

During the previous year we have completed and extended our nucleotide sequencing studies of the genomic S RNA from

lymphocytic choriomeningitis virus (Armstrong strain). Coding regions within the S RNA were identified using the synthetic peptide approach and we have generated several computer comparisons between the predicted amino acid sequences for the LCMV structural proteins and other arenavirus structural proteins (Southern et al., Virol. 157:145-155, 1987; Southern and Bishop, Current Topics Microbiol. and Immunol. 133:19-39, 1987). Conserved and divergent regions can be readily identified; the extent of conservation is significantly higher in GP-2 than in GP-1 for the sequences currently available (Pichinde, Lassa, LCM WE, LCM Arm).

In additional sequencing studies, we have collaborated with Maria Salvato and Michael Oldstone to compare the cDNA sequence from clones with the sequence derived from direct RNA sequencing. There were only two nucleotide differences in the two sequences--one did not change the corresponding acid and the other changed the predicted amino acid from alanine to arginine at GP position 177 (Salvato et al., Virol., submitted). The essential confirmation of the sequence by two independent approaches provides considerable confidence in the accuracy of the sequence. A limitation with the cDNA clones arises because each clone is derived from an individual RNA molecule and therefore may not be representative of the viral RNA population.

We have now identified several cDNA clones from the genomic L RNA segment of LCMV. Currently, the clone nucleotide sequence information covers about 4 kb, representing approximately 50% of the L segment. We have predicted parts of the amino acid sequence for L-encoded proteins and have synthesized short peptides corresponding to these potential viral proteins. Anti-peptide antibodies have then been used in Western blotting experiments to identify a high molecular L protein that is present both in purified virions and intracellular viral RNP complexes (Singh et al., Virol. 161:448-456, 1987). The anti-peptide antibodies have provided the first mono-specific reagents to study the distribution of L proteins during infection.

In order to understand the molecular events associated with the progression from acute to persistent arenavirus infection, we have studied the temporal relationship of LCMV replication and transcription during acute infection. At early times, NP mRNA and progeny genomic sense RNAs begin to accumulate simultaneously; the GP mRNA accumulates more slowly within the cells (Fuller-Pace and Southern, Virol. 162, in press, 1988). In other experiments, we have established an *in vitro* assay for the viral RNA-dependent RNA polymerase activity. We anticipate combining all of this information to allow systematic comparisons of intracellular RNAs, proteins and polymerase activities in acute and persistent infection to explore the molecular basis of persistent arenavirus infection.

During our visit to Ft. Detrick in June 1987, Drs. Southern and Buchmeier performed RNA extractions of Lassa viral genomic RNA for purposes of molecular cloning. The extracted RNA was agarose

gel purified (see Appendix 2), safety tested and when proven non-infectious was sent to California where an initial round of cDNA cloning was done. We are currently analyzing the cDNAs produced.

D. In vivo protective efficacy of Vaccinia constructs containing LCMV NP and GP-C genes

We have obtained Vaccinia vectors containing various constructions of the LCMV GP-C and NP genes and have performed two pilot experiments to test their protective efficacy in guinea pigs against an LCMV WE challenge inoculation. The results of these experiments are summarized in Table 1.

Table 1

Protective Efficacy of vaccinia LCMV Constructs Against LCMV-WE Challenge in Guinea Pigs

Experiment 1

| <u>Immunize with</u> | <u>N</u> | <u>Challenge</u> | <u>Mortality</u> |
|--|----------|------------------|------------------|
| LCMV Arm (10^4 pfu ip) | 3 | WE (10^4 pfu) | 0/3 |
| VV _{SC11} (without insert) (10^8 pfu Id) | 3 | WE | 3/3 |
| VV _{B5} (full length WE, GP-C insert) (10^8 pfu Id) | 8 | WE | 8/8 |

Experiment 2

| <u>Immunize with</u> | <u>N</u> | <u>Challenge</u> | <u>Mortality</u> |
|--|----------|------------------|------------------|
| LCMV Arm (10^4 pfu ip) | 2 | WE | 0/2 |
| VV _{SC11} | 3 | WE | 3/3 |
| VV _{NP} (full length NP) (10^8 pfu Id) | 8 | WE | 8/8 |
| VV _{NP} & VV _{GP7} (5×10^7 pfu each Id) | 4 | WE | 4/4 |

In neither case did we see evidence of significant protection against in vivo challenge with WE.

The results of these experiments were disappointing to us and will force a reconsideration of our vaccinia approaches in the coming year. We intend to study in some detail the antibody and CMI responses of guinea pigs to vaccinia-LCMV constructs as well

as to the obviously protective LCMV-Arm infection. ELISA results on the guinea pigs in experiment 1 showed only two to four-fold rises in anti-LCMV titers three weeks after vaccinia-LCMV inoculation.

We attempted as well to measure lymphocyte proliferative activity against LCMV antigens, but were consistently hampered by high levels of spontaneous proliferation. We will try in the coming year to iron out these problems and generate a clearer picture of the responses to primary inoculation with VV constructs.

It should be noted that others working with these same constructs have been unable to generate primary LCMV CTL responses in spite of the fact that cells infected with the VV LVMC constructs are efficiently recognized and killed by primed CTL (J. L. Whitton, personal communication).

E. Plans for the coming year

- 1) We will continue our efforts to define immunogenic regions of the arenavirus glycoproteins with the aim of identifying important epitopes for neutralization and other biological functions.
- 2) Existing and additional cDNA clones of Lassa virus will be characterized. We hope also to begin cloning of Mopeia virus.
- 3) Immune responses of guinea pigs to primary inoculation with vaccinia virus constructs containing LCMV genetic information will be further characterized.

Appendix 1: Publications supported by this document

Published Papers

Buchmeier, M.J., P.J. Southern, B.S. Parekh, M.K. Wooddell, and M.B.A. Oldstone. 1987. Site specific antibodies define a cleavage site conserved among arenavirus GP-C glycoproteins. *J. Virol.* 61:982-985.

Buchmeier, M.J. and B. S. Parekh. 1987. Protein structure and expression among arenaviruses. *Curr. Topics Microbiol. Immunol.* 133:41-57.

Southern, P.J., M.K. Singh, Y. Riviere, D.R. Jacoby, M.J. Buchmeier, and M.B.A. Oldstone. 1987. Molecular characterization of the genomic S RNA segment from lymphocytic choriomeningitis virus. *Virology* 157:145-155.

Singh, M. K., F. V. Fuller-Pace, M. J. Buchmeier, and P. J. Southern. 1987. Analysis of the genomic L RNA segment from lymphocytic choriomeningitis virus. *Virology* 161:448-456.

Papers in Press

Weber, E. L., and M. J. Buchmeier. Fine mapping of a heptapeptide containing a group specific antigen conserved among arenavirus glycoproteins. *Virology*, in press, 1988.

Buchmeier, M. J., K. E. Wright, E. L. Weber, and B.S. Parekh. Structure and expression of arenavirus proteins. In *Proceedings of the First International Symposium on Immunobiology and Pathogenesis of Persistent Virus Infections*, April 26-29, 1987, in press.

Fuller-Pace, F. V., and P. J. Southern. Temporal analysis of transcription and replication during acute infection with lymphocytic choriomeningitis virus. *Virology*, in press, 1988.

Appendix 2: Method of RNA extraction and gel separation

Extraction of Lassa Virus RNA. 6/87.

Purified virus pellets stored in SW41 centrifuge tubes at -70°C.
0.2 ml virus/tube in TNE (10 mM Tris HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0).

Virus samples transferred into screw-cap eppendorf tubes already containing phenol.
(Phenol was pre-equilibrated in 0.5 M Tris HCl pH 8.0 plus x1 TNE).

Samples extracted x3 sequentially with phenol.

Heavy protein interface after the first extraction - this was left behind as the aqueous layer (containing the released viral RNA) was transferred to a new tube for the next extraction. No protein interface was visible after the second and third phenol extractions (Footnote 1).

Final aqueous solution adjusted to 0.25 M NaCl final concentration and the RNAs were precipitated with 2 volumes of 95% ethanol at -70°C for 10 minutes. The RNAs were pelleted by centrifugation for 10 minutes in the microcentrifuge and then the pellets were washed with 95% ethanol and re-centrifuged (Footnote 2).

Excess ethanol was removed by inversion and then the RNAs were redissolved in a total volume of 200 µl of sterile H₂O.

20 µl of this material mixed with 20 µl gel dye (10 mM NaPO₄ pH 6.5, 50% glycerol, 0.25% SDS, 0.1% bromophenol blue and 0.1% xylene cyanol FF) and brought out through the dunk tank.

Remaining RNA stored at -70°C in P4 lab.

Footnote 1: All phenol solutions were combined and back extracted once with an equal volume of TNE. After centrifugation, the aqueous layer was removed into a new tube, adjusted to 0.25 M NaCl final concentration and left to precipitate at -20°C (P4) with 2 volumes of 95% ethanol.

Footnote 2: All ethanol supernatants were saved and stored at -20°C (P4).

Gel Analysis

The first sample to be brought out (approx. 10% of total material) was dried on a vacuum desiccator to remove traces of ethanol and then resuspended with 10 µl of sterile H₂O. Electrophoresis on an agarose minigel showed bands in the Lassa RNA corresponding to viral genomic L and S species and host 28S and 18S ribosomal RNA species.

The bulk of the Lassa RNA (approx. 220 µl) was transferred into a new screw-cap vial containing 200 µl of gel loading buffer (see above) and brought out through the dunk tank. (The original tube, containing the RNA sample, was retained for safety testing.) The RNA sample was dried on a vacuum desiccator to remove traces of ethanol, resuspended in sterile H₂O and electrophoresed on an agarose mini-gel. Bands were located by ethidium bromide fluorescence and excised from the gel. Samples from each gel band (approx. 5%) were retained for safety testing and the remainder of the gel slices were stored at -70°C.

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